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Effect of ten amino acids on elimination of acrylamide in a model reaction system

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Ten (10) amino acids (asparagine, aspartic acid, cysteine, glycine, glutamic acid, glutamine, methionine, leucine, tyrosine and lysine) were used to test their effect on elimination of acrylamide, when co-heating of 10 μmol of acrylamide respectively with 0.1 mmol of amino acids at 160°C for 15 min, acrylamide was eliminated from 7.4% (asparagine) to 94.4% (cysteine) at natural pH, and 11.4% (glutamic acid) to 94.8% (cysteine) at pH 7.0. It was found that cysteine, glysine and lysine showed much higher reactivity with acrylamide than the other seven amino acids. When the molar ratios of amino acid to acrylamide were increased to 60:1 for cysteine and glycine, and 80:1 for lysine, acrylamide in the reaction system was almost eliminated. The elimination efficiencies of the three amino acids for acrylamide were enhanced by addition of glucose. Liquid chromatography/time-of-flight mass spectroscopy showed that cysteine, glycine, and lysine adducted with two molecules of acrylamide, while the other amino acids except for glutamic acid (no adduct compounds were found) just adducted with one mole of acrylamide. This finding may partly explain why cysteine, glycine and lysine showed high capacity to reduce acrylamide content in high-temperature processing food.

Key words: Acrylamide, adduct, amino acids.

INTRODUCTION

Acrylamide is a neurotoxic compound and probable carcinogen found at significant levels in carbohydrate-rich processed foods. It is largely derived from heat-induced reactions between the amino group of the free amino acid asparagine and the carbonyl group of reducing sugars, such as glucose, during baking and frying (Friedman, 2003; Koutsidis et al., 2009).

Addition of other amino acids has been proposed as a mitigation strategy to reduce the levels of acrylamide in crisps, flat breads and bread crust (Vinci et al., 2012). Glycine, lysine and cysteine have received particular attention as additives that could potentially reduce acrylamide formation by either competing for available Maillard reaction inter-mediates or reacting with acrylamide itself through Michael addition (Adams et al., 2010; Friedman and Levin, 2008; Ou et al., 2010). Zamora et al. (2010) speculated that each molecule of amino acid could form adducts with one or two molecules of acrylamide, but they did not identify the later one.

In addition to the intended addition of amino acids into food, detectable amounts of various free amino acids have been found in food raw materials like potato, wheat and rice (Elmore et al., 2005; Mustafa et al., 2007; Saikusa et al., 1994).

In order to investigate the efficiency of different amino acids to eliminate acrylamide, 10 amino acids were used and reacted with acrylamide in model reaction system; and the type of adducts formed were determined using liquid chromatography/time -of-flight mass spectroscopy (LC/TOF-MS).

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MATERIALS AND METHODS

Chemicals

Asparagine, aspartic acid, cysteine, glycine, glutamic acid, glutamine, methionine, leucine, tyrosine, lysine and glucose were purchased from Aladdin Reagents Database Inc. (Shanghai, China) and were of 99% purity. The acrylamide standard (>99.8%) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). HPLC-grade methanol was purchased from J.T. Baker (USA).

Effect of amino acids on elimination of acrylamide and determination of their adducts

Aqueous solutions (4 mL) containing 10 μmol acrylamide and 0.1 mmol of a single amino acid species (asparagine, aspartic acid, cysteine, glycine, glutamic acid, glutamine, methionine, leucine, tyrosine and lysine), were heated in sealed stainless test tubes at 160°C for 15 min by incubation in a polyethylene glycol (MW 600) bath (Ou et al., 2008). The pH values of the samples were kept unadjusted or adjusted to 7.0 using 0.2 mol/L phosphate buffer before heating. At the end of reaction, the test tubes were cooled in an ice bath. The reaction solutions were decanted, and 6 mL of deionised water was added to wash the test tubes. The reaction and wash solutions were combined and then centrifuged at 8000 ×g for 20 min. Acrylamide and its amino acid adducts in the supernatant were characterised and quantified by HPLC (Figure 1), ESI-MS, and time-of-flight mass spectrometry (TOF-MS).

Effect of cysteine, glycine and lysine on elimination of acrylamide

Mixtures (4 mL) containing 10 μmol acrylamide and different amounts (0.1, 0.2, 0.4, 0.6 and 0.8 mmol) of single amino acids (glycine, cysteine and lysine), with or without the addition of 0.1 mmol of glucose, were reacted in sealed stainless test tubes at 160°C for 15 min. The pH was either left unadjusted or adjusted to 7.0 using 0.2 mol/L phosphate buffer. At the end of the reaction, the test tubes were cooled in an ice bath. The reaction solutions were decanted, and 6 mL of deionised water was added to each test tube. Specific reaction and wash solutions were combined and then centrifuged at 8000 ×*g* for 20 min. The residual acrylamide was determined by HPLC.

Determination of acrylamide

Quantification of acrylamide was performed on a Shimadzu LC-20AT system (Kyoto, Japan) equipped with a diode array detector and LC-solution software. An Agilent Zorbax SB Aq C_{18} column (4.6 mm x 250 mm, 5 μm; Waldbronn, Germany) was selected for acrylamide analysis. The injection volume was 5 μL. Elution was carried out at a flow rate of 0.5 mL/min under isocratic conditions at 30°C using 5% methanol aqueous solution as the mobile phase. Acrylamide was detected at 205 nm.

Determination of the adducts between amino acid and acrylamide using TOF-MS

The adducts of amino acids with acrylamide were determined using an Agilent 6210 LC/TOF-MS with an Agilent Zorbax SB-C_{18} column (2.1mm × 30mm, 3.5 μm). The TOF-MS operation parameters were as follows: drying gas (N_2) at 9 L/min flow rate; drying gas temperature, 300°C; nebulising gas (N_2) pressure, 30 psi; capillary voltage, 3500 V; skimmer voltage, 65 V and fragmentor voltage, 300 V. Elution was performed at a flow rate of 0.4 mL/min under isocratic conditions and using 10% methanol aqueous solution as the mobile phase. TOF-MS scanning results were collected as centroids from 100 to 2000 m/z. Two reference mass compounds, a lock mass solution including purine ($C_5H_4N_4$ at m/z 121.050873) and hexakis (1H, 1H, 3H-tetrafluoropentoxy)-phosphazene ($C_{18}H_{18}O_6N_3P_3F_{24}$ at m/z 922.009798) were used to perform real-time lock mass correction. ESI positive or negative ions were used to determine the molecular weight of the components in the products and identify amino acid and acrylamide adducts.

RESULTS AND DISCUSSION

Effect of amino acids on acrylamide elimination

Except for asparagine, aspartic acid and glutamine, the other amino acids tested obviously decreased acrylamide levels at natural pH (allowing the pH to change as the reaction proceeded). In addition, cysteine showed the highest efficiency in reducing acrylamide, with acrylamide disappearance of 94.6%, followed by glycine (72.1%) and lysine (69.6%). The starting pH of aspartic acid - acrylamide, glutamic acid -acrylamide, glutamine - acrylamide and lysine-acrylamide system were 2.85, 3.10, 4.54 and 9.67, respectively, and that of the rest systems were between 6.01 and 6.90. A buffered model reaction system is designed to eliminate impact of pH differences between each amino acid on test results. When reaction pH was kept constant at 7.0, the amount of residual acrylamide fell slightly in contrast with that of unbuffered reaction system (Table 1). The results showed that pH difference did not influence greatly on amino acid-acrylamide reaction.

Among the amino acids tested, cysteine, glycine and lysine were most effective on eliminating acrylamide but they could not completely deplete free acrylamide when the molar ratio was kept at 10:1. In the later research, different molar ratios of amino acids to acrylamide, from 10: 1 to 80: 1, were tested to investigate whether or not increasing amino acid concentration could further eliminate acrylamide. Acrylamide was decreased gradually as the increase of amino acid addition amount (Table 2). When the molar ratio rose to 60: 1 for cysteine and glycine or 80:1 for lysine, the amount of residual acrylamide in the reaction system was reduced to below the detection limit.

During the Maillard reaction, cysteine, glycine and lysine concentrations would be reduced while reacting with reducing sugars. To investigate whether Maillard reaction would influence the elimination effect of acrylamide by the three amino acids, glucose was added into the reaction system. The results showed that addition of glucose did not decrease but instead increased the acrylamide-reducing efficiency of these amino acids, except for lysine (Table 3).

Formation of adducts between amino acids and acrylamide

Acrylamide contains an α,β-unsaturated group which can

Table 1. Effect of amino acids on elimination of acrylamide in a model system added with 10 μmol of acrylamide and 0.1 mmol of amino acid heating at 160°C for 15 min.

Amino acid	Blank	Asn[a]	Asp	Cys	Gly	Glu	Gln	Met	Leu	Tyr	Lys
Residual acrylamide at nature pH (μmol)	10.28 ±0.23[b]	9.26±0.45	9.21±0.16	0.56±0.12	2.87±0.34	7.48±0.26	9.23±0.36	7.92±0.35	8.47±0.37	8.48±0.15	3.13±0.21
Residual acrylamide at pH 7.0 (μmol)	10.11±0.21	8.78±0.17	9.15±0.23	0.52±0.05	2.48±0.11	8.86±0.13	8.74±0.26	7.24±0.06	8.12±0.13	7.65±0.12	2.83±0.06

[a]AA, amino acid; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gly, glycine; Glu, glutamic acid; Gln, glutamine; Met, methionine; Leu, leucine; Tyr, tyrosine; Lys, Lysine;[b]Means ± SD (n=3).

Table 2. Effect of different amount of cysteine, glycine and lysine on elimination of 10 μmol of acrylamide in 4 ml of aqueous solution heating at pH 7.0 for 15 min.

Amino acid	Residual acrylamide (μmol) after reaction with different amount of amino acids				
	0.1 mmol	0.2 mmol	0.4 mmol	0.6 mmol	0.8 mmol
Cysteine	0.50±0.03[a]	0.42±0.02	0.21±0.03	ND[b]	ND
Glycine	2.53±0.02	1.78±0.02	0.63±0.01	ND	ND
Lysine	3.23±0.04	1.81±0.04	1.23±0.03	0.67±0.01	ND

[a]Means ± SD (n=3); [b]Not determined (below 0.05 μg/mL).

Table 3. Effect of different amount of cysteine, glycine and lysine on elimination of 10 μmol of acrylamide heating with 0.1 mmol glucose at pH 7.0 for 15 min.

Amino acid	Residual acrylamide (μmol) after reacting with different amount of amino acids				
	0.1 mmol	0.2 mmol	0.4 mmol	0.6 mmol	0.8 mmol
Cysteine	0.46±0.04	0.31±0.07	0.14±0.02	ND	ND
Glycine	1.58±0.07	1.07±0.12	0.31±0.03	ND	ND
Lysine	3.68±0.16	2.73±0.13	1.27±0.07	0.13±0.01	ND

[a]Means ± SD(n=3); [b]Not determined (below 0.05 μg/ml).

reacts with different kinds of nucleophiles including amino acids through Michael addition. According to Zamora et al. (2010), one molecule of some amino acids can react with one molecule of acrylamide through the Michael reaction to form compound 1, and they speculated that one molecule of acrylamide can adduct with two molecules of some amino acids to form compound 2. However, they did not detect the compound 2 during the reaction of acrylamide with butylamine, glycine and lysine, respectively.

In this research, compound 1 was detected in the reaction mixture of acrylamide with all tested amino acids except for glutamic acid by TOF-MS (Table 4), and the compound 2 was successfully detected in the reaction system of acrylamide with cysteine, glycine and lysine, respectively (Table 4). Moreover, the ions at m/z 205 and 203 which

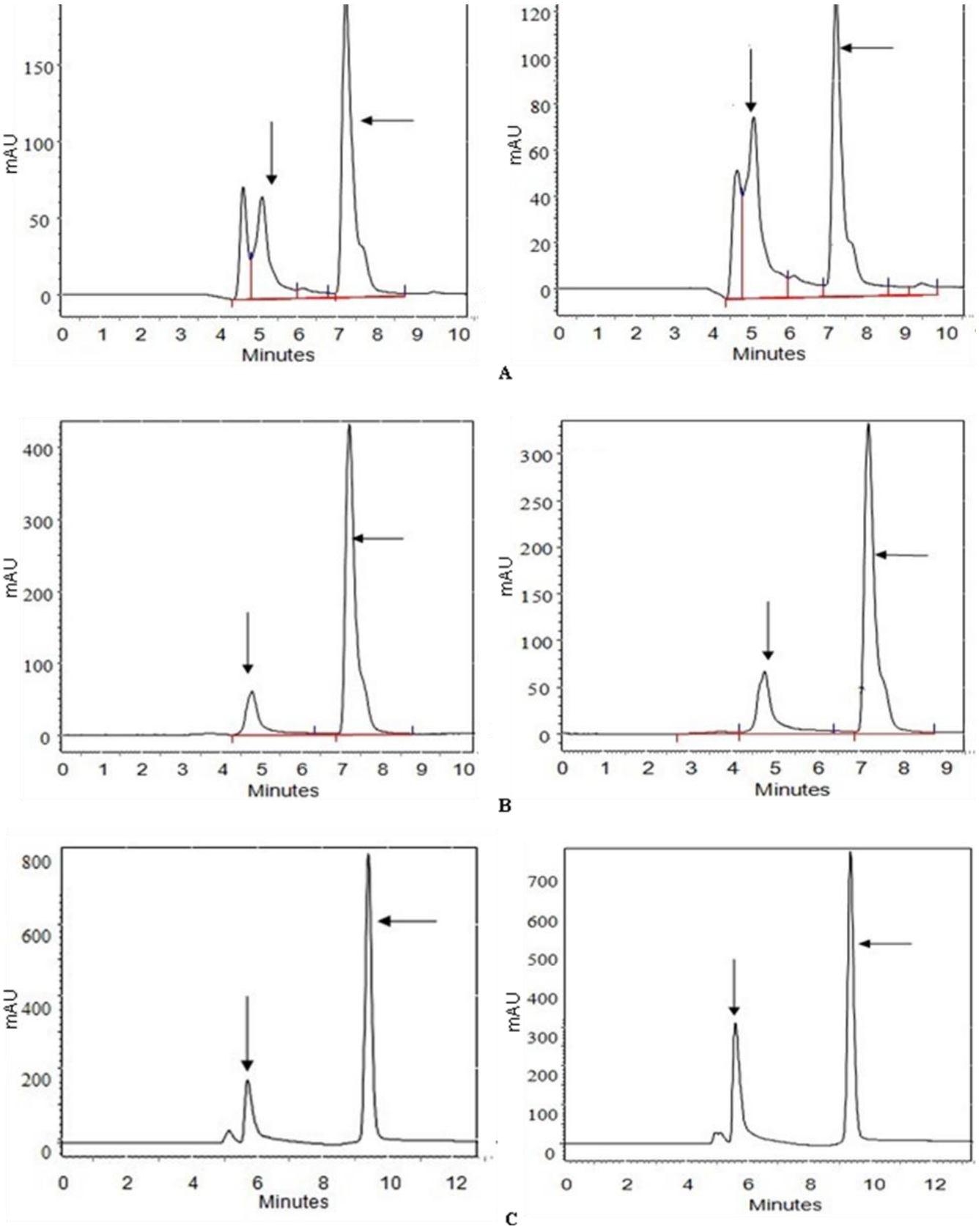

Figure 1. HPLC of the reaction products from 10 μmol acrylamide reacted with 0.1 mmol (left) and 0.2 mmol (right) cysteine (A), glycine (B), and lysine (C). The vertical arrow indicates the peak for the adduct formed from the reaction of acrylamide with amino acids. The horizontal arrow indicates the peak for acrylamide.

Table 4. Detected accurate mass and predicted chemical formulas of adduct 1 and adduct 2 by TOF-MS.

AA[a]	Ion	Calc m/z	m/z	Diff (ppm)	Formula
Asn	$(M+H)^+$	204.09788	204.09762	1.28	$C_7H_{13}N_3O_4$
Asp	$(M+H)^+$	205.0819	205.08143	2.29	$C_7H_{12}N_2O_5$
Cys	$(M+H)^+$	193.06414	193.06478	-3.35	$C_6H_{12}N_2O_3S$
Cys	$(M+H)^+$	264.10125	264.10160	-1.33	$C_9H_{17}N_3O_4S$
Gly	$(M-H)^-$	145.06187	145.06188	-0.13	$C_5H_{10}N_2O_3$
Gly	$(M-H)^-$	216.09898	216.0989	0.4	$C_8H_{15}N_3O_4$
Glu	-	-	-	-	-
Gln	$(M+H)^+$	218.1141	218.1124	-7.8	$C_8H_{15}N_3O_4$
Met	$(M+H)^+$	221.09544	221.09531	0.57	$C_8H_{16}N_2O_3S$
Leu	$(M+H)^+$	203.13902	203.13944	-2.09	$C_9H_{18}N_2O_3$
Tyr	$(M+H)^+$	253.11828	253.11844	-0.61	$C_{12}H_{16}N_2O_4$
Lys	$(M+Na)^+$	204.09788	204.09762	1.28	$C_9H_{19}N_3O_3$
Lys	$(M+H)^+$	289.18703	289.18707	-0.13	$C_{12}H_{24}N_4O_4$

[a]The full name was listed in the footnote of Table 1.

correspond respectively to the $[M+H]^+$ and $[M-H]^-$ of the adduct for aspartic with acrylamide were detected by ESI-MS in asparagine-acrylamide reaction system, suggesting that, during the heating process, asparagine may be hydrolyzed to aspartic acid which then reacts with acrylamide.

The TOF-MS results may partly explain why cysteine, glycine and lysine showed high capacity to reduce acrylamide in food system. As reported by Zamora et al. (2010), the compoud 1 is not stable and easy to decompose. Thus, the amino acids that can only form compound 1 with acrylamide would show low elimination efficiency for acrylamide because of decomposition of compound 1. However, cysteine, glycine and lysine can react with acrylamide to form compound 2, the later compound may be more stable than the compound 1, thus make these amino acids show higher elimination efficiency for acrylamide. According to Koutsidis et al. (2009), cysteine and glycine, which were able to form compound 2 tested in our research, could form relatively more amino acid-acrylamide adducts when compared with another 3 amino acids.

Moreover, analysis of the products of three amino acids reacting with acrylamide by HPLC at 205 nm revealed that, there were one or two peaks with shorter retention time than acrylamide in the chromatogram. The peak area increased as the peak area of acrylamide decreased (Figure 1), HPLC-MS results of the reaction products between acrylamide and cysteine, lysine and glycine respectively confirmed that they were adduct 1 and adduct 2. This finding may encourage us to detect the adducts in food system.

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